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Adenoviral vectors for prodrug activation-based gene therapy for cancer

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Abstract

Cancer cell heterogeneity is a common feature - both between patients diagnosed with the same cancer and within an individual patient's tumor - and leads to widely different response rates to cancer therapies and the potential for the emergence of drug resistance. Diverse therapeutic approaches have been developed to combat the complexity of cancer, including individual treatment modalities designed to target tumor heterogeneity. This review discusses adenoviral vectors and how they can be modified to replicate in a cancer-specific manner and deliver therapeutic genes under multi-tiered regulation to target tumor heterogeneity, including heterogeneity associated with cancer stem cell-like subpopulations. Strategies that allow for combination of prodrug-activation gene therapy with a novel replication-conditional, heterogeneous tumor-targeting adenovirus are discussed, as are the benefits of using adenoviral vectors as tumor-targeting oncolytic vectors. While the anticancer activity of many adenoviral vectors has been well established in preclinical studies, only limited successes have been achieved in the clinic, indicating a need for further improvements in activity, specificity, tumor cell delivery and avoidance of immunogenicity.

Keywords

Cancer gene therapy; cytochrome P450 prodrug activation; replication-conditional adenovirus; oncolytic adenovirus; tumor heterogeneity

Replication-conditional oncolytic adenoviruses for cancer treatment

Oncolytic virotherapy offers a cancer therapeutic strategy whereby viral genomes are modified for specific therapeutic applications. Replication-conditional adenoviruses have intrinsic anticancer activity and the potential for therapeutic gene delivery [1, 2]. Moreover, modifications of the adenoviral genome can be introduced to impart tumor-cell selectivity and reduce the risk of viral spread and toxicity in primary host tissues.

One class of modifications is comprised of adenoviral gene deletions, which may be introduced to create either a replication-defective adenovirus or a replication-conditional adenovirus. Replication-defective adenoviruses can be generated by deleting the entire adenoviral E1 gene region, which eliminates E1A, an immediate early viral oncoprotein required for adenoviral replication. Other viral gene deletions impart cancer-specific (conditional) replication, thereby killing cancer cells in a selective manner. Deletion of the adenoviral *E1B-55kDa* gene yields a virus that replicates in a tumor-specific manner based

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on the p53 status of the infected cell [3]. E1B-55kDa protein binds to, sequesters, and facilitates degradation of p53, thereby ablating both the tumor suppressor and pro-apoptotic activity of p53 [2, 4, 5]. Adenoviruses that lack E1B-55kDa protein cannot block p53-induced apoptosis in normal (host) cells, where p53 is expressed in an active, functional form. Consequently, host cells infected with E1B-55kDa-deficient adenovirus die in a p53-dependent manner before the virus can repackage itself and spread. E1B-55kDa deletion may confer replication selectivity in tumor cells as compared to normal tissues based on the mutation and/or deficiency of p53 pathway factors in the majority of human cancers [3, 6, 7]. One example is the *E1B-55 kDa* deleted, oncolytic adenovirus ONYX-015, which has been evaluated in phase II and phase III clinical trials [8]. ONYX-015 was initially thought to replicate only in p53-deficient cells [9], however, later studies showed that it can also replicate in cells bearing a wild-type *p53* gene [10], suggesting a risk of permissive replication in normal tissues. The leakiness of this regulation may be explained by the finding that E1B-55 kDa protein plays a role in host cell protein synthesis shut-off and late adenoviral mRNA export, which can be phenocopied by other factors, both in tumor cells and in certain normal cells [11]. These observations indicate a need to improve upon the safety of *E1B-55 kDa*-deleted oncolytic adenoviruses.

A second E1B region gene, *E1B-19 kDa*, inhibits apoptosis in a manner similar to Bcl-2; it binds to and represses the pro-apoptotic transcription repressor Btf [12, 13], and it inactivates the pro-apoptotic Bcl-2 family member Bax [14]. *E1B-19 kDa* deletion yields an adenovirus with increased oncolytic potency, as infected cells lyse more quickly, increasing the rate of virus spread [15]. Both E1B genes delay adenovirus-induced cell death long enough for completion of virus replication and repackaging; these anti-apoptotic genes are therefore not required for viral replication in many cancer cells, where apoptosis is already suppressed [16, 17]. Thus, deletion of either (or both) E1B genes yields an oncolytic adenovirus with improved anti-tumor activity [18–20]. However, deletion of both E1B genes has been found to shift the mechanism of cell lysis from a strictly apoptosis-independent mechanism [21] to one that involves apoptosis [22, 23]. Furthermore, even in cancer cells, if the extent of viral-induced apoptosis is too high, as with a cancer-cell replication conditional, E1B region-deleted adenovirus, viral lysis of host cancer cells can occur before the completion of adenoviral replication and repackaging, thereby decreasing adenoviral titer [22]. As such, the E1B genes are both important for effective virus replication and spread even in cancer tissues and should not be deleted. An alternative strategy is to express the E1B genes in a cancer-specific manner using cancer-specific promoter elements, as discussed below.

The adenoviral E3 gene region can inhibit immune-mediated apoptosis [3, 6]. E3 is thus considered unnecessary for adenovirus replication in cancer cells and has been deleted in many adenoviruses engineered for cancer therapy. However, it is important to consider that the E3 region contains the adenoviral death protein (ADP), which facilitates efficient adenoviral lysis of infected host cells [24].

The adenoviral *E1A* gene is required for adenoviral replication. It is the first RNA transcribed from the adenoviral genome, within ~1 hr of viral infection [25, 26]. E1A codes for an oncoprotein, that is required for transformation and signaling of downstream events, including activation, transcription and replication of the rest of the adenoviral genome. E1A interacts with various cellular peptides, including the retinoblastoma gene product, p105-RB, which is important in oncogenesis [27].

Gene therapy schemes that incorporate the adenoviral *E1A* gene inhibit tumor angiogenesis [28]. In addition, E1A protein sensitizes tumor cells to both chemotherapy and radiation [29–31] and induces a p53-dependent increases in apoptosis [16, 28, 32]. Consequently,

adenovirus lacking the anti-apoptotic activities of both E1B proteins and expressing high levels of E1A induce E1A-dependent early host cell apoptosis and cytolysis, thereby interrupting viral progeny maturation and decreasing viral spread [22].

Using cancer-specific promoters to impart cancer cell specificity

Tissue-specific promoter elements may be used to control expression of the E1A oncoprotein in a tumor-specific manner, resulting in tumor cell-specific adenoviral transcriptional activation and replication [2, 33, 34]. Two such cancer-specific regulatory elements are discussed below.

Human telomerase

Human telomerase (hTERT) has received much attention due to the relationship between telomere length/activity and cellular senescence and immortalization. Telomere shortening occurs in dividing somatic cells and serves as an internal mitotic/cell cycle clock; when telomeres are reduced to a minimum critical length, cell senescence occurs. Telomerase is an enzymatic ribonucleoprotein that synthesizes the telomeric repeats, 5'-TTAGGG-3' [35], found at chromosomal ends. Telomerase is instrumental in preventing telomere shortening and senescence, and can confer immortality to malignant cells, where it is often highly expressed [36]. Telomerase activity is dependent on an RNA template (hTER) [37] and includes a reverse transcriptase (hTERT) and telomerase associated proteins [38]. Telomerase activity requires only the RNA component and hTERT. Both hTER and hTERT are required for reconstitution of telomerase activity *in vitro* [39, 40]. In contrast to hTER, which is widely expressed in embryonic and somatic tissues, hTERT is highly regulated and is not detectable in most somatic cells [41, 42]. However, hTERT expression parallels telomerase activity during the course of cellular differentiation and neoplastic transformation [43, 44].

hTERT is active in 85–90% of tumor tissues and is detectable in the early stages of malignancy [45]. The hTERT promoter is inactive in most normal, host tissues but displays high activity in a majority of human cancers [46, 47]. Telomerase is thus considered to be an ideal tumor-specific regulator of oncolytic adenoviruses [23, 48]. A 320 bp hTERT promoter fragment contains the sequences required to recapitulate high telomerase expression in cancer cells [49–51] (Fig. 1A). These sequences include five SP1 binding sites, which in normal somatic cells repress telomerase expression [52], two E-Boxes containing c-Myc and Max binding sites [53], and an activating enhancer-binding protein-2 (AP-2) binding site, which induces hTERT expression [54, 55]. AP-2 β has also been shown to support tumor-specific reactivation of hTERT in human lung cancer cells [56].

A minimal hTERT promoter can be used to limit adenoviral E1A expression to hTERT-positive cancer cells. hTERT promoter-regulated adenoviruses induce cytopathic effects in tumor cells comparable to those of wild-type adenovirus [57, 58]. In addition, the hTERT promoter has been utilized in several gene therapy models [48]. Telomerase promoter-regulated adenoviruses that retain one or both of the E1B gene products (E1B-55 kDa and/or E1B-19 kDa) have been investigated [48].

The potential of combining the increased oncolytic activity conferred by deletion of both E1B genes with the tumor cell-specificity imparted by the use of an hTERT core promoter regulating the adenoviral *E1A* gene has been investigated [23]. Tumor cells infected with the resulting adenovirus, Adeno-hTERT-E1A, express significantly higher levels of E1A oncoprotein, and show enhanced lysis and an earlier and higher apoptotic index than cells infected with the replicating adenovirus ONYX-015, which has a wild-type E1A promoter [23]. Furthermore, Adeno-hTERT-E1A displays a substantial increase in tumor cell

specificity compared to the oncolytic adenovirus ONYX-015, as evidenced by a dramatic decrease in Adeno-hTERT-E1A genome replication, E1A expression, and viral cycle completion in primary human hepatocytes [23].

hTERT has been successfully used as a cancer-specific promoter to regulate therapeutic transgenes delivered by replication-deficient adenoviral vectors. Delivery of a replication-deficient adenovirus utilizing hTERT-driven expression of thymosin $\beta(10)$ induced reactive oxygen species production and apoptosis of ovarian cancer cells [59]. Replication-deficient adenovirus incorporating an hTERT-driven TRAIL death ligand cassette showed improved anti-tumor activity against adenoid cystic carcinoma [60]. Furthermore, adenoviral vectors encoding hTERT-driven expression of either Bax or TRAIL were shown to sensitize pancreatic tumors to gemcitabine treatment, yielding improved regression responses and increased survival in animal models [61].

DF3/Mucin1

DF3/Mucin1 (MUC1) is a mucin-like glycoprotein that is overexpressed in ~75% of all human solid tumors, including many late-stage cancers, including those of the pancreas, prostate, breast, ovaries and lungs [62–66]. DF3/MUC1 protein epitopes and mRNA levels are often altered, showing moderate to aberrant increases in expression or, less frequently, loss of expression in adenocarcinomas when compared to corresponding normal tissues [64]. DF3/MUC1 regulates intracellular oxidant levels and apoptosis in response to oxidative stress [67]. Interestingly, DF3/MUC1 expression correlates with the degree of breast cancer differentiation and estrogen receptor status [68]. DF3/MUC1 functions as an oncogene [69], plays a role in cellular adhesion, invasion and metastasis [63], is a determinant of resistance to Herceptin [70], and its initial altered expression is considered a hallmark of premalignant cells [64]. DF3/MUC1 expression has been correlated with poor prognosis [71], and DF3/MUC1-positive stage IV pancreatic cancer patients have a decreased survival rate compared to DF3/MUC1-negative patients [63]. DF3/MUC1 expression is also related to the cancer progression. For example, DF3/MUC1 expression increases through stage III pancreatic cancer, and peaks in stage IV [63]. DF3/MUC1 is also associated with MCF-7 breast cancer tumor-forming stem/progenitor cell-like side populations [72] and growth regulation of human pluripotent stem cells [73]. For these reasons, the DF3/MUC1 promoter and enhancer are a good choice when designing replication-conditional adenoviruses [74–77]. A DF3/MUC1 promoter has been used to drive adenoviral E1A expression; this virus replicates in a manner comparable to wild-type adenovirus in breast cancer cells, whereas replication is decreased in DF3-negative cell lines [1].

Deletion analysis of the DF3/MUC1 promoter has shown that high-level expression can be achieved using 720 nt of upstream sequence (Fig. 1A). Two transcription factor binding sites play key roles in DF3/MUC1 transcriptional regulation: an Sp1 site and an E-box [78, 79], with the upstream Sp1 site being important for epithelium-specific MUC1 expression [80].

Other cancer-selective promoters that have been used to drive adenoviral E1A expression include tyrosinase promoter, L-plastin promoter for estrogen-dependent cancers, prostate-specific antigen promoter, osteocalcin promoter (active in prostate tumors and in bone metastases), E2F-1 promoter, hypoxia responsive element, the radiation inducible early growth response gene 1 promoter, and the cyclooxygenase-2 promoter [2].

Adenoviral based gene therapy

Adenoviral genomes can be modified by the insertion of foreign genes with cancer therapeutic activity. Adenoviral genomes typically range from 30 to 38 kbp in size, with the capsid coat of a viral particle able to package up to ~40 kbp, i.e., only approximately 1.8 kb

more genetic material than the wild-type virus genome. Non-essential viral genes can be deleted to increase the capacity for delivery of therapeutic genes, including immunomodulatory genes and other genes with therapeutic potential [2]. These adenoviral gene modifications can be introduced to engineer tumor cell selectively-replicating adenoviruses, as discussed above, and can be combined with the delivery of specific genes for the purpose of gene therapy. One such approach is the introduction of genes coding for enzymes that activate a cancer chemotherapeutic prodrug (prodrug-activating enzyme genes), as discussed below.

Use of adenoviruses for prodrug activation gene therapy

Chemotherapy employing cytotoxic drugs is a mainstay of cancer therapy. However, conventional cancer chemotherapy can have limited utility, as it is often not specific to cancer cells, resulting in substantial toxicity in normal host tissues. One approach to reduce non-specific toxicity, termed gene-directed enzyme prodrug therapy (GDEPT), involves the delivery of a chemosensitization or suicide gene directly to tumor cells to increase their sensitivity to drugs that would otherwise be non-cytotoxic or less cytotoxic [81–85]. GDEPT has potential for treating tumors that are non-responsive or poorly responsive to conventional cancer chemotherapeutic treatments. This strategy also has promise for low systemic toxicity as a result of tumor-localized prodrug activation following targeted gene delivery with either viral or non-viral vectors (Fig. 2) [1, 2, 33].

Genes coding for prodrug-activating enzymes may be delivered to tumor cells using a variety of vectors, including replication-deficient adenoviral vectors; alternatively, tumor-cell selective delivery may be achieved using a replication-conditional adenoviral vector [2, 86–88]. Tumor cells infected with such a virus acquire the capacity to convert a prodrug to its active cytotoxic metabolite locally, i.e., within the tumor, resulting in an overall increase in tumor cell death at a given drug dosage [89]. As such, GDEPT can sensitize tumor cells to chemotherapeutic prodrugs. This strategy may allow for a reduction in the drug dose while maintaining overall therapeutic efficacy, thereby decreasing systemic side effects towards critical host tissues [90, 91].

In one prodrug-enzyme system, the cancer chemotherapeutic prodrug cyclophosphamide is metabolized to its active form, 4-hydroxycyclophosphamide (4-OH-CPA), by enzymes of the CYP2B subfamily of cytochromes P450. These P450 enzymes are most highly expressed in the liver, where cyclophosphamide metabolism leads to the production and systemic distribution of 4-OH-CPA and its cytotoxic derivatives. To specifically localize activated drug metabolites in tumors, directed intratumoral expression of a cyclophosphamide-activating *CYP2B* gene—not normally expressed at significant levels by cancer cells—increases intratumoral drug activation and anti-cancer activity [83, 92–94]. As such, GDEPT using prodrug-activating P450 genes may be useful in treating a broad spectrum of cancers, including breast cancer, melanoma, metastatic liver cancer, and pancreatic cancer [93, 95–97].

An important feature of GDEPT is the cytotoxic bystander effect, whereby activated, membrane-permeable drug metabolites, such as 4-OH-CPA, can readily diffuse across cell membranes, enabling the cytotoxic drug to reach tumor cells surrounding the tumor ‘factory cells’ that harbor the prodrug-activating genes and produce the active metabolite (Fig. 3A). Bystander killing is an essential feature of any GDEPT system, insofar as it helps circumvent the requirement (unachievable using current gene delivery technologies) to transduce 100% of the target tumor cell population with the therapeutic gene. Tumor cell apoptosis induced by 4-OH-CPA is a slow process, taking about 2 to 3 days to be manifest [98], which prolongs the life of the factory cells and thus increased bystander activity [83].

Arming a replication-conditional adenovirus with a prodrug-activating P450 gene in combination with its redox partner, P450 reductase, can, in principle, be used to combat a broad spectrum of cancers [83, 84].

Several other prodrug/prodrug-activating enzyme combinations may be useful for cancer treatment. These include ganciclovir in combination with herpes simplex virus thymidine kinase (TK), 5-fluorocytosine (5-FC) with bacterial cytosine deaminase (CD), and irinotecan (CPT-11) with carboxylesterase [99]. While prodrug metabolites can synergize with the oncolytic effect of conditionally replicating adenoviruses, drugs that inhibit adenoviral replication, such as topoisomerase inhibitors [100], should be avoided. These other prodrug-enzyme systems have been utilized in conjunction with replication-competent adenoviral therapies [2, 89] and with lentiviral vectors [101], which integrate and therefore have the potential of disrupt genes in the host genome.

Enhancement of GDEPT bystander activity by the pan-caspase inhibitor p35

In an alternative gene therapeutic approach to increase tumor cell killing, pro-apoptotic factors can be used to augment drug-induced tumor cell apoptosis. Thus the pro-apoptotic factors, such as Bax, p53, Trail and various caspases, have been investigated in both preclinical and clinical studies, either alone or in combination with traditional chemotherapy [102]. A major drawback of this strategy, however, is that it does not elicit bystander cell killing, and consequently, pro-apoptotic genes must be delivered to nearly 100% of cells in a given tumor cell population *in vivo* in order to elicit a sustained anti-tumor response. Moreover, such pro-apoptotic strategies are not compatible with GDEPT, because they undermine the bystander killing effect by inducing early death of the prodrug-activating factory cells [102].

An alternative, albeit counter intuitive approach combines GDEPT with the introduction of anti-apoptotic factors in order to prolong the life of the prodrug-metabolizing factory tumor cell and thereby increase overall cytotoxic drug production [102]. The baculovirus protein p35, a 35-kDa single chain broad-spectrum pan caspase inhibitor [103, 104], was found to be the most effective of several caspase inhibitors tested in delaying the death of 9L gliosarcoma cells stably expressing P450 and treated with cyclophosphamide [105]. Importantly, p35 delayed but did not block the ultimate death of the transduced tumor cells (Fig. 3B). This strategy was exemplified using a replication-deficient adenoviral vector that delivered the cyclophosphamide-activating P450 enzyme CYP2B6 in combination with its redox partner P450 reductase and p35 [106]. Importantly, adenoviral delivery of p35 did not introduce drug resistance. The concept of utilizing an anti-apoptotic factor to delay the death of prodrug-activating factory cells may be extended to other anti-apoptotic factors and other prodrug-activating gene therapy systems.

Adenovirus helper effect

Replication-competent adenoviruses can be used to supply viral replication and packaging proteins *in trans* to facilitate the spread and expression of replication-deficient adenoviruses harboring a therapeutic transgene (Fig. 4) [107]. Such helper virus systems have been used to increase anticancer activities of combination therapies involving replication-competent adenoviruses, cancer gene therapy, and chemotherapy. Replication-competent adenoviruses, such as ONYX-015 and the closely related (E3 region wild-type) derivative ONYX-017 [107, 108], have been combined with replication-defective adenoviruses to facilitate delivery of P450 and other therapeutic genes to tumor cells *in vivo* [107, 109]. Replication-competent adenoviruses that contain a cancer cell-selective promoter regulating the

expression of adenoviral E1A have also been studied for their potential as helper viruses [22]. One hTERT-driven adenovirus employing dual E1B gene deletions, Adeno-hTERT-E1A, exhibited increased E1A expression, which in turn increased the helper activity of this adenovirus. However, an adenovirus with a second, independent E1A cassette regulated by the core DF3/MUC1 promoter, Adeno-DF3-E1A/hTERT-E1A, resulted in an increase in E1A expression to levels that increased apoptosis, leading to premature host cell death, a decrease in adenoviral titer and loss of viral helper ability [22]. In the same study, helper activity was evaluated for the oncolytic adenovirus ONYX-015, which retains the *E1B-19 kDa* gene, and in contrast to Adeno-hTERT-E1A [23], induced little or no detectable tumor cell apoptosis. Despite the inclusion of the *E1B-19kDa* gene, ONYX-015 exhibited less helper virus activity toward a replication-deficient adenovirus encoding the cyclophosphamide-activating P450 enzyme CYP2B11 than Adeno-hTERT-E1A. Taken together with the loss of helper virus activity in the case of Adeno-DF3-E1A/hTERT-E1A, these results demonstrate the importance of balancing E1A production with the extent and the timing of virus-induced cytolysis. Prodrug enzyme-based gene therapy strategies are most effective in the context of delayed, rather than accelerated death of tumor factory cells that express the prodrug-activating enzyme [102].

Interestingly, when the anti-apoptotic factor p35 was used to inhibit cyclophosphamide-induced apoptosis and delay tumor cell death, it did not interfere with the helper effect of the replication-conditional adenovirus ONYX-017. This finding is consistent with the apoptosis-independent mechanism of host cell death [21] upon expression of the adenoviral death protein [110], which is retained in the E3 region of ONYX-017 [88]. When ONYX-017 was used as a helper virus for a replication-deficient adenovirus encoding both CYP2B6 and p35, bystander cytotoxicity was enhanced to a greater extent than when used for a replication-deficient adenovirus expressing CYP2B6 alone, Adeno-2B6. Overall tumor cell killing was more extensive when tumor cells were infected with ONYX-017 in combination with Adeno-2B6/p35 as compared to Adeno-2B6 [106].

These findings highlight the benefit of using a two-virus helper system for increasing the capacity for transgene delivery and therapeutic activity. In the context of a one-virus system, balancing p35-mediated protection and viral lysis and spread from host cells would need to be considered. While replication-competent adenoviruses that directly encode therapeutic transgenes have been investigated [2], a two-virus system may be particularly useful in cases where the total transgene size is too large to engineer into a single replicating virus. Furthermore, the combination of conditionally replicating and non-replicating adenoviruses could be ideal for GDEPT, due to the synergistic effect of combining replicating virus-induced tumor cytolysis with intratumoral activation of chemotherapeutic prodrugs conferred by a replication-defective virus.

Targeting tumor heterogeneity

Genome-wide expression profiling studies have shown that tumor cell heterogeneity is widespread [111, 112]. Such heterogeneity may arise by mechanisms such as cell differentiation, tumor progression and clonal expansion [113]. Different tumor microenvironments contribute to tumor heterogeneity by affecting the differentiation and genetic evolution of tumor cell subpopulations, which may impact both intrinsic and acquired resistance to various therapeutics [114, 115]. Moreover, heterogeneity between primary tumors and their metastatic lesions is common and further complicates cancer treatment. Studies of tumor cell heterogeneity related to stem/progenitor cell-like niches, cell differentiation states, and cycling versus non-cycling cell status [72] highlight the difficulty in effectively targeting the entire tumor cell population in a given patient.

Expression of cancer-specific genes, whose promoters are used to confer cancer cell specificity to adenoviral infection (discussed above) can also be heterogeneous. For instance, hTERT expression can vary widely, ranging from undetectable in benign mesenchymal lesions and low-grade soft tissue carcinomas to being detectable in ~50% of intermediate-/high-grade soft tissue carcinomas [116]. Telomerase activity and hTERT mRNA levels show substantial intratumoral heterogeneity, even within histologically similar regions [116]. Other studies show that telomerase activity varies among patients with glioblastoma multiforme [117–119], prostatic carcinoma [120], breast cancer [121], and hepatocellular carcinoma [122]. Heterogeneity of cancer cell telomere lengths within individual tumors has also been reported. Intratumoral heterogeneity was seen in up to 27% of paired samples taken from two different regions of individual sarcomas [116]. Shorter and longer telomeres were observed more often in low/intermediate-grade and high-grade soft tissue carcinomas, respectively. In human patients, hTERT was also enriched in metastatic lesions from primary breast tumors [123]. *hTERT* transcription is up regulated by hypoxia, suggesting differential oxygenation as one mechanism for the variation of hTERT expression within a given tumor [124].

Though DF3/MUC1 was initially associated with breast carcinomas, DF3/Muc1 is increasingly recognized as being expressed in many types of cancer. Heterogeneity of expression was seen in an analysis of DF3/MUC1 over a panel of 14 human breast carcinoma cell lines *in vitro* and in 12 of those cell lines when grown as xenografts *in vivo*. Considerable variability in DF3/MUC1 expression was observed, not only between cell lines but also between cells within a given tumor cell line [71, 125]. DF3/MUC1 expression is markedly heterogeneous following malignant transformation [71]. The mechanisms underlying this heterogeneity are not well understood, and it has yet to be determined whether it reflects differences in transcriptional activation [71]. Moreover, DF3/MUC1 gene and protein structures are highly unstable; they include a variable number (typically 30 to 90) of 20 amino acid tandem repeats. This instability contributes to allelic variation of this polymorphic protein. DF3/MUC1 contains two protein domains that associate as a heterodimer in its cell surface anchored form. These are translated as a single polypeptide that is then cleaved into an NH₂-terminal domain containing hydrophobic signal sequences and a more stable COOH-terminus including a transmembrane domain and a cytoplasmic tail [69, 78]. The resultant two polypeptides associate to yield a heterodimer anchored at the cell surface. In a study of primary human breast carcinomas, 70 of 110 breast tumor DNA samples exhibited DF3/MUC1 heterogeneity associated with loss of heterozygosity (29% of cases) and an increase in copy number of one allele (8% of cases). These genetic changes did not necessarily map to genomic regions surrounding the *DF3/MUC1* locus, indicating that *DF3/MUC1* itself is affected by mutations at high frequencies [126]. Heterogeneity of DF3/MUC1 expression is also seen at different stages of cancer differentiation and progression [62, 63, 65]. Notably, DF3/MUC1 expression was increased in MCF-7 breast and U251 brain cancer stem cell-associated subpopulations [22, 72].

When multiple tumor-specific promoters are used to regulate adenoviral replication, there is an increased likelihood that viral anti-tumor activity will not be lost due to tumor heterogeneity. One such adenovirus, Adeno-DF3-E1A/hTERT-E1A, contains two separate *E1A* cassettes driven by two different promoters, DF3/MUC1 and hTERT [22]. This dual promoter-regulated adenovirus shows increased activity across a panel of cancer cell lines with varying levels of DF3/MUC1 and hTERT, when compared to the single hTERT-promoter adenovirus Adeno-hTERT-E1A. Furthermore, the 5-fold lower expression of hTERT in U251 tumor cell subpopulations enriched for tumor stem- and progenitor-like cells (U251 holoclones) as compared to the overall U251 cell population led to a decrease in oncolytic activity following infection with the single promoter hTERT adenovirus [22]. In contrast, in the case of the dual E1A cassette virus Adeno-DF3-E1A/hTERT-E1A, the 10-

fold elevation of DF3/MUC1 levels in U251 holoclones was associated with increased oncolytic activity [22]. This work complements efforts to use adenoviral vectors to target cancer stem cell populations [127, 128].

Tumor heterogeneity associated with variable conditions within the tumor microenvironment may contribute to the ineffectiveness *in vivo* of single-promoter regulated adenoviruses that exhibit potent oncolytic activity *in vitro*. U251 solid tumors grown *in vivo* in *scid* mice exhibit 10-fold lower levels of *hTERT* expression, as compared to U251 cells grown in culture; however, these same tumors showed a 40-fold increase in expression of *DF3/MUC1* [22]. When the anti-tumor activities of the single *hTERT*-regulated adenovirus, Adeno-*hTERT*-E1A, and the dual DF3/*hTERT*-regulated adenovirus, Adeno-DF3-E1A/*hTERT*-E1A, were examined, only the dual DF3/*hTERT* adenovirus exhibited significant anti-tumor activity [22]. Incorporation of a DF3/MUC1 promoter, which shows increased activity in the many cancers where DF3/MUC1 is overexpressed [62–66], may be beneficial in treating cancers, such as gliosarcomas, that are not normally associated with high DF3/MUC1 expression in cultured cell lines. Large increases in DF3/MUC1 expression and large decreases in *hTERT* expression were also observed in A549 lung tumor xenografts, as compared to cultured A549 cells, and MDA-MB-231 breast cancer xenografts also exhibited increased DF3/MUC1 levels as compared to cultured MDA-MB-231 cells [22]. While the causes of these changes in DF3/MUC1 and *hTERT* expression in solid tumor xenografts are unknown, the increases in DF3/MUC1 expression may be related to its roles in tumor angiogenesis and extracellular matrix remodeling [66]. These findings highlight the potential advantage of using a dual promoter regulated adenovirus to circumvent tumor microenvironment-associated factors that decrease adenoviral promoter activity and thus viral anti-tumor activity.

Given these findings, dual promoter-regulated adenoviruses have the potential for improved clinical responses over single promoter-regulated adenoviruses. An *hTERT* promoter-regulated adenovirus, Telomelysin, was recently evaluated in a phase I clinical trial and exhibited limited anti-tumor activity with viral replication observed only in a small subset of patients [129]. While it was not examined in the context of the anti-tumor activity of Telomelysin, *hTERT* heterogeneity was found across cancers within the patient population [129] and could help explain the limited anticancer activity. Other adenoviruses use a single dual-specificity promoter to regulate E1A expression in response to multiple stimuli, e.g., estrogens and hypoxia [76].

Future Directions

Improving the replicative capacity and anti-tumor efficacy of adenoviral vectors

A potential issue in the way that both the previously mentioned *hTERT* alone and dual DF3/*hTERT* adenoviruses replicate is the hybrid killing mechanism of viral lysis and premature induced host cell death *via* apoptosis, which has been shown to decrease viral titer in certain cancer cell lines, such as A549 and U251 [22]. This is likely a direct consequence of deleting the adenoviral E1B region. To eliminate premature host-cell lysis to rescue viral replication and titer, one might reconstitute the wild-type adenovirus E1B as well as ADP in the adenoviral E3 region to re-establish an apoptosis-independent killing mechanism in these adenoviruses. Viral replication would then be relegated strictly to cancer cells by the cancer-specific promoters DF3 and *hTERT*. In order to retain the feature of using E1B gene region deletion to impart additional cancer-cell specificity to viral replication, an internal ribosomal entry sequence (IRES) could be used to place the *E1B-55 kDa* and *E1B-19 kDa* genes under the control of the DF3 and/or *hTERT* promoters. In doing so, the anti-apoptotic E1B proteins would only be expressed in a cancer-selective manner when either DF3 or *hTERT* promoter is activated. One consequence, however, of having one long transcript running

from the adenoviral E1A into the E1B region is that the formation of these longer transcripts may be diminished due to their increased length. In addition, one observed consequence of utilizing an IRES is that the expression of the second gene in the overall transcript can be diminished compared to the first gene [94]. However, as long as enough of the E1B transcript and E1B proteins are produced, this potential decrease may not be important. One such virus where E1A and the E1B genes were placed under control of an hTERT promoter using a spanning IRES (Telomelysin) in fact exhibited enhanced lysis [130]. Another strategy might be to determine whether apoptosis suppression will increase the ability of E1B-deleted viruses to successfully replicate to completion. One might co-infect cancer cells with either of the two replication-conditional adenoviruses previously mentioned (hTERT or DF3/hTERT) in combination with Adeno-p35, a replication-deficient adenovirus that expresses the baculovirus pan-caspase inhibitor p35 [106]. If intracellular viral content as well as the amount of functional, released viral progeny increase then this would show that apoptosis suppression is necessary to increase or rather rescue the replication potential of these two E1B region-deleted adenoviruses. However, one caveat is that many adenoviral proteins have evolved to be multi-functional to accommodate the limited genomic sequence that can be packaged into the viral protein capsid. As such, if the adenoviral E1B proteins carry out other functions necessary for viral replication, apoptosis suppression may be necessary but not sufficient to restore their replication to wild-type levels.

Immunogenic obstacles to the successful implementation of adenoviral therapies

Adenoviral clearance by the immune system is a major obstacle to the clinical use of therapeutic adenoviruses. Many studies of oncolytic adenoviruses have been performed in adaptive immunodeficient mouse models, such as *scid* and *nude* mice, where the innate immune system is still intact. Adenoviruses may be cleared by circulating macrophages and other innate immune cells, such as dendritic cells, natural killer cells and granulocytes [131, 132]. The liver is highly infectable by adenovirus, and has a large reservoir of Kupffer cells (liver macrophages) and natural killer cells [133], which may contribute to the systemic clearance of circulating adenovirus. These innate immune responses are elicited by adenoviral capsid coat proteins and do not require viral transcription and replication [131]. Thus, both replication-deficient and replication-competent adenoviruses may be cleared by innate immune cells. As such, innate immune responses as well as adaptive immune responses in immune competent hosts [134] may negatively impact the ability of these viruses to elicit anti-tumor effects by diminishing the longevity of their replicative capacity *in vivo*, leading to more rapid clearance of replicating adenoviruses. Thus, the potential impact of the extent and type of immune compromise between mouse strains on adenoviral efficacy should be explored. For instance, NOD-scid-gamma (NSG) mice, which have greatly diminished innate immunity, might be used to test the anticancer potency of these adenoviruses in the same tumor xenograft models previously used in *scid*, *nude*, or fully immune competent mice. While these models are artificial due to varying levels of compromised immunity, it would be instructive to determine the extent to which the anticancer potential of these adenoviral vectors is compromised by different tiers (innate vs. adaptive) of immune interference. If so, strategies to reduce this interference in immune competent hosts, discussed below, should be the main focus in improving anti-cancer adenoviral therapeutics.

The ability of macrophages and other innate immune cells to phagocytose adenoviral particles is not blocked by modifications to the fiber-knob regions involved in adenoviral internalization [131]. Rather, adenoviral surface antigens must be masked to prevent immunogenic effects [135, 136]. This may be accomplished by PEGylation [137] or by modification of viral coat proteins to eliminate immunogenicity [2]. In the case of replication-competent adenoviruses, one novel strategy is to infect cells in culture and then

inject these pre-infected carrier cells, now containing replication-conditional adenoviruses, into mice with established tumors. This approach circumvents the problems of hepatocyte sequestration and immune clearance seen with circulating adenovirus [138, 139]. Several cell types, including primary T lymphocytes, cytokine-induced killer cells, progenitor cells, and pre-irradiated immortalized cell lines, have the ability to home to primary cancer beds as well as metastatic tumors within 24 to 72 hr [138, 140]. As long as the carrier cells make their way to the tumors before they are lysed, both immunogenic interference and non-specific infection (i.e., infection of non-cancerous host tissue) can be minimized [138]. Systemic delivery of adenovirus packaged in ultrasound contrast agent microbubbles may also help avoid premature viral clearance and immune intervention [141]. If viral activity is eventually detected by the immune system, it will do so directly in the cancer bed itself, potentially eliciting an anti-tumor immune response. Cell- or material-based carriers also allow for increased tumor infiltration and perfusion by replication-competent adenoviruses. One issue with adenoviral trafficking within tumors is that there is only about 30–40 nm of space within the extracellular matrix for movement of viral particles. This is a particular problem for adenoviral spread, because the diameter of adenovirus capsids is ~80–100 nm in size.

One potential drawback to the use in human cancer treatment of adenoviruses that encode therapeutic transgenes isolated from other species is the potential to elicit immune responses to the encoded foreign antigens. Such responses might lead to elimination of those cells that are successfully transduced with the therapeutic gene and thereby decrease the effectiveness of the gene therapeutic strategy. An alternative approach is to introduce an endogenous, mammalian anti-apoptotic factor, specifically one that is present in humans, in order to avoid immune system clearance of transduced factory cells [102]. However, if the goal is to treat tumors that are already known to suppress or evade immune attack, another possibility is to deliver effective anti-apoptotic factors, regardless of their origin, using a replication-competent adenovirus. Introduction of a foreign antigen, such as the pan-caspase inhibitor p35, might elicit an anti-tumor immune response *via* an immune cell type that is not tumor-suppressed or, at the very least, the introduction of p35 will not be cleared by tumor-suppressed immune cells and will therefore be useful for GDEPT.

Conclusion

Replication-conditional adenoviruses engineered for cancer cell specificity have shown promise as stand-alone anti-tumor agents with the additional potential for therapeutic gene delivery. Various strategies have been used to improve the tumor-specificity of anti-tumor viruses while reducing toxicity to non-malignant host tissues. However, even with these improvements, tumor-targeted adenoviruses have had limited success in the clinic. Development of additional strategies is thus required; these include prolongation of GDEPT-associated bystander activity using anti-apoptotic factors such as p35, incorporation of multiple promoter regulatory elements to target tumor heterogeneity, and improvement of adenoviral delivery to tumors while masking viral particle immunogenicity.

Synergistic anti-tumor responses may be achieved by utilizing multiple therapeutic modalities, such as cancer gene therapy combined with oncolytic adenovirus infection and radiochemotherapy. This, in turn, could allow for a reduction in the dosage of one or more of the components in the combination therapy, thereby reducing overall toxicity of the treatment. The heterogeneous nature of most cancers necessitates the employment of combination therapies, including viral strategies incorporating multiple cancer-specific regulatory elements, in an effort to eradicate critical tumor cell subpopulations, including cancer stem-like cell populations.

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Abbreviations

4-OH-CPA	4-hydroxycyclophosphamide
ADP	adenoviral death protein
AP-2	activating enhancer-binding protein-2
CMV	cytomegalovirus
CPA	cyclophosphamide
E1A	early adenoviral region 1A
GDEPT	gene-directed enzyme prodrug therapy
hTERT	human telomerase
PARP	poly (ADP-ribose) polymerase
pfu	plaque forming units
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

References

1. Bauerschmitz GJ, Barker SD, Hemminki A. Adenoviral gene therapy for cancer: from vectors to targeted and replication competent agents (review). *Int J Oncol.* 2002; 21(6):1161–1174. [PubMed: 12429964]
2. Jounaidi Y, Doloff JC, Waxman DJ. Conditionally Replicating Adenoviruses for Cancer Treatment. *Curr Cancer Drug Targets.* 2007; 7(3):285–301. [PubMed: 17504125]
3. Kasuya H, Takeda S, Nomoto S, Nakao A. The potential of oncolytic virus therapy for pancreatic cancer. *Cancer Gene Ther.* 2005; 12(9):725–736. [PubMed: 15818382]
4. Grand RJ, Parkhill J, Szeszak T, Rookes SM, Roberts S, Gallimore PH. Definition of a major p53 binding site on Ad2E1B58K protein and a possible nuclear localization signal on the Ad12E1B54K protein. *Oncogene.* 1999; 18(4):955–965. [PubMed: 10023671]
5. Steegenga WT, Riteco N, Jochemsen AG, Fallaux FJ, Bos JL. The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene.* 1998; 16(3):349–357. [PubMed: 9467960]
6. Liu TC, Kim D. Viruses with deletions in antiapoptotic genes as potential oncolytic agents. *Oncogene.* 2005; 24(40):6069–6079. [PubMed: 16155595]
7. O'Shea CC, Soria C, Bagus B, McCormick F. Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell.* 2005; 8(1):61–74. [PubMed: 16023599]
8. Crompton AM, Kim DH. From ONYX-015 to armed vaccinia viruses: the education and evolution of oncolytic virus development. *Curr Cancer Drug Targets.* 2007; 7(2):133–139. [PubMed: 17346104]
9. Bischoff JR, Kim DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, McCormick F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells [see comments]. *Science.* 1996; 274(5286):5373–5376.
10. Dix BR, Edwards SJ, Braithwaite AW. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway. *J Virol.* 2001; 75(12):5443–5447. [PubMed: 11356950]

11. O'Shea CC, Johnson L, Bagus B, Choi S, Nicholas C, Shen A, Boyle L, Pandey K, Soria C, Kunich J, Shen Y, Habets G, Ginzinger D, McCormick F. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell*. 2004; 6(6):611–623. [PubMed: 15607965]
12. Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. *Mol Cell Biol*. 1999; 19(6):4390–4404. [PubMed: 10330179]
13. Imazu T, Shimizu S, Tagami S, Matsushima M, Nakamura Y, Miki T, Okuyama A, Tsujimoto Y. Bcl-2/E1B 19 kDa-interacting protein 3-like protein (Bnip3L) interacts with bcl-2/Bcl-xL and induces apoptosis by altering mitochondrial membrane permeability. *Oncogene*. 1999; 18(32): 4523–4529. [PubMed: 10467396]
14. Han J, Sabbatini P, Perez D, Rao L, Modha D, White E. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev*. 1996; 10(4):461–477. [PubMed: 8600029]
15. Liu TC, Hallden G, Wang Y, Brooks G, Francis J, Lemoine N, Kirn D. An E1B-19 kDa gene deletion mutant adenovirus demonstrates tumor necrosis factor-enhanced cancer selectivity and enhanced oncolytic potency. *Mol Ther*. 2004; 9(6):786–803. [PubMed: 15194046]
16. Rao XM, Tseng MT, Zheng X, Dong Y, Jamshidi-Parsian A, Thompson TC, Brenner MK, McMasters KM, Zhou HS. E1A-induced apoptosis does not prevent replication of adenoviruses with deletion of E1b in majority of infected cancer cells. *Cancer Gene Ther*. 2004; 11(9):585–593. [PubMed: 15338010]
17. Rao XM, Zheng X, Waigel S, Zacharias W, McMasters KM, Zhou HS. Gene expression profiles of normal human lung cells affected by adenoviral E1B. *Virology*. 2006; 350(2):418–428. [PubMed: 16542696]
18. Harrison D, Sauthoff H, Heitner S, Jagirdar J, Rom WN, Hay JG. Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved--deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. *Hum Gene Ther*. 2001; 12(10):1323–1332. [PubMed: 11440625]
19. Duque PM, Alonso C, Sanchez-Prieto R, Lleonart M, Martinez C, de Buitrago GG, Cano A, Quintanilla M, Ramon y, Cajal S. Adenovirus lacking the 19-kDa and 55-kDa E1B genes exerts a marked cytotoxic effect in human malignant cells. *Cancer Gene Ther*. 1999; 6(6):554–563. [PubMed: 10608352]
20. Kim J, Kim JH, Choi KJ, Kim PH, Yun CO. E1A- and E1B-Double mutant replicating adenovirus elicits enhanced oncolytic and antitumor effects. *Hum Gene Ther*. 2007; 18(9):773–786. [PubMed: 17725410]
21. Abou, El; Hassan, MA.; van der Meulen-Muileman, I.; Abbas, S.; Kruij, FA. Conditionally replicating adenoviruses kill tumor cells via a basic apoptotic machinery-independent mechanism that resembles necrosis-like programmed cell death. *J Virol*. 2004; 78(22):12243–12251. [PubMed: 15507611]
22. Doloff JC, Jounaidi Y, Waxman DJ. Dual E1A oncolytic adenovirus: targeting tumor heterogeneity with two independent cancer-specific promoter elements, DF3/MUC1 and hTERT. *Cancer Gene Ther*. 2011; 18(3):153–166. [PubMed: 20865021]
23. Doloff JC, Waxman DJ, Jounaidi Y. Human telomerase reverse transcriptase promoter-driven oncolytic adenovirus with E1B-19 kDa and E1B-55 kDa gene deletions. *Hum Gene Ther*. 2008; 19(12):1383–1400. [PubMed: 18771358]
24. Tollefson AE, Ryerse JS, Scaria A, Hermiston TW, Wold WS. The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. *Virology*. 1996; 220(1):152–162. [PubMed: 8659107]
25. Lewis JB, Mathews MB. Control of adenovirus early gene expression: a class of immediate early products. *Cell*. 1980; 21(1):303–313. [PubMed: 7407913]
26. Nevins JR, Ginsberg HS, Blanchard JM, Wilson MC, Darnell JE Jr. Regulation of the primary expression of the early adenovirus transcription units. *J Virol*. 1979; 32(3):727–733. [PubMed: 513202]
27. Harlow E, Whyte P, Franza BR Jr, Schley C. Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol Cell Biol*. 1986; 6(5):1579–1589. [PubMed: 2431282]

28. Zhou Z, Zhou RR, Guan H, Bucana CD, Kleinerman ES. E1A gene therapy inhibits angiogenesis in a Ewing's sarcoma animal model. *Mol Cancer Ther.* 2003; 2(12):1313–1319. [PubMed: 14707272]
29. Deissler H, Opalka B. Therapeutic transfer of DNA encoding adenoviral E1A. *Recent Pat Anticancer Drug Discov.* 2007; 2(1):1–10. [PubMed: 18221050]
30. Liao Y, Yu D, Hung MC. Novel approaches for chemosensitization of breast cancer cells: the E1A story. *Adv Exp Med Biol.* 2007; 608:144–169. [PubMed: 17993239]
31. Martin-Duque P, Sanchez-Prieto R, Romero J, Martinez-Lamparero A, Cebrian-Sagarriga S, Guinea-Viniegra J, Dominguez C, Lleonart M, Cano A, Quintanilla M, Ramon YCS. In vivo radiosensitizing effect of the adenovirus E1A gene in murine and human malignant tumors. *Int J Oncol.* 1999; 15(6):1163–1168. [PubMed: 10568823]
32. Hale TK, Braithwaite AW. The adenovirus oncoprotein E1a stimulates binding of transcription factor ETF to transcriptionally activate the p53 gene. *J Biol Chem.* 1999; 274(34):23777–23786. [PubMed: 10446138]
33. Everts B, van der Poel HG. Replication-selective oncolytic viruses in the treatment of cancer. *Cancer Gene Ther.* 2005; 12(2):141–161. [PubMed: 15472714]
34. Nevins JR. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell.* 1981; 26(2 Pt 2):213–220. [PubMed: 7332929]
35. Greider CW. Telomere length regulation. *Annu Rev Biochem.* 1996; 65:337–365. [PubMed: 8811183]
36. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000; 100(1):57–70. [PubMed: 10647931]
37. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene.* 2001; 269(1-2):1–12. [PubMed: 11376932]
38. Stewart SA, Weinberg RA. Telomeres: cancer to human aging. *Annu Rev Cell Dev Biol.* 2006; 22:531–557. [PubMed: 16824017]
39. Beattie TL, Zhou W, Robinson MO, Harrington L. Reconstitution of human telomerase activity in vitro. *Curr Biol.* 1998; 8(3):177–180. [PubMed: 9443919]
40. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, Andrews WH, Wright WE, Shay JW, Harley CB, Morin GB. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nat Genet.* 1997; 17(4):498–502. [PubMed: 9398860]
41. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell.* 1997; 90(4):785–795. [PubMed: 9288757]
42. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. Telomerase catalytic subunit homologs from fission yeast and human. *Science.* 1997; 277(5328):955–959. [PubMed: 9252327]
43. Takakura M, Kyo S, Kanaya T, Tanaka M, Inoue M. Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. *Cancer Res.* 1998; 58(7):1558–1561. [PubMed: 9537264]
44. Wu A, Ichihashi M, Ueda M. Correlation of the expression of human telomerase subunits with telomerase activity in normal skin and skin tumors. *Cancer.* 1999; 86(10):2038–2044. [PubMed: 10570429]
45. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer.* 1997; 33(5):787–791. [PubMed: 9282118]
46. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science.* 1994; 266(5193):2011–2015. [PubMed: 7605428]
47. Mo Y, Gan Y, Song S, Johnston J, Xiao X, Wientjes MG, Au JL. Simultaneous targeting of telomeres and telomerase as a cancer therapeutic approach. *Cancer Res.* 2003; 63(3):579–585. [PubMed: 12566299]

48. Wirth T, Kuhnel F, Kubicka S. Telomerase-dependent gene therapy. *Curr Mol Med*. 2005; 5(2): 243–251. [PubMed: 15974879]
49. Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res*. 1999; 59(4):826–830. [PubMed: 10029071]
50. Kyo S, Takakura M, Taira T, Kanaya T, Itoh H, Yutsudo M, Ariga H, Inoue M. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res*. 2000; 28(3):669–677. [PubMed: 10637317]
51. Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res*. 1999; 59(3):551–557. [PubMed: 9973199]
52. Won J, Yim J, Kim TK. Sp1 and Sp3 recruit histone deacetylase to repress transcription of human telomerase reverse transcriptase (hTERT) promoter in normal human somatic cells. *J Biol Chem*. 2002; 277(41):38230–38238. [PubMed: 12151407]
53. Oh S, Song YH, Kim UJ, Yim J, Kim TK. In vivo and in vitro analyses of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. *Biochem Biophys Res Commun*. 1999; 263(2):361–365. [PubMed: 10491298]
54. Ma H, Urquidí V, Wong J, Kleeman J, Goodison S. Telomerase reverse transcriptase promoter regulation during myogenic differentiation of human RD rhabdomyosarcoma cells. *Mol Cancer Res*. 2003; 1(10):739–746. [PubMed: 12939399]
55. Kirkpatrick KL, Ogunkolade W, Elkak AE, Bustin S, Jenkins P, Ghilchick M, Newbold RF, Mokbel K. hTERT expression in human breast cancer and non-cancerous breast tissue: correlation with tumour stage and c-Myc expression. *Breast Cancer Res Treat*. 2003; 77(3):277–284. [PubMed: 12602927]
56. Deng WG, Jayachandran G, Wu G, Xu K, Roth JA, Ji L. Tumor-specific activation of human telomerase reverses transcriptase promoter activity by activating enhancer-binding protein-2beta in human lung cancer cells. *J Biol Chem*. 2007; 282(36):26460–26470. [PubMed: 17630431]
57. Uchino J, Takayama K, Harada A, Kawakami Y, Inoue H, Curiel DT, Nakanishi Y. Infectivity enhanced, hTERT promoter-based conditionally replicative adenoviruses are useful for SCLC treatment. *Cancer Gene Ther*. 2005; 12(9):737–748. [PubMed: 15861179]
58. Wirth T, Zender L, Schulte B, Mundt B, Plentz R, Rudolph KL, Manns M, Kubicka S, Kuhnel F. A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res*. 2003; 63(12):3181–3188. [PubMed: 12810646]
59. Kim YC, Kim BG, Lee JH. Thymosin beta(10) Expression Driven by the Human TERT Promoter Induces Ovarian Cancer-Specific Apoptosis through ROS Production. *PLoS One*. 2012; 7(5):e35399. [PubMed: 22623951]
60. Zang G, Miao L, Mu Y, Qiao C, Liu J, Ke X, Zheng C, Sun H. Adenoviral mediated transduction of adenoid cystic carcinoma by human TRAIL gene driven with hTERT tumor specific promoter induces apoptosis. *Cancer Biol Ther*. 2009; 8(10):966–972. [PubMed: 19587531]
61. Wack S, Rejiba S, Parmentier C, Aprahamian M, Hajri A. Telomerase transcriptional targeting of inducible Bax/TRAIL gene therapy improves gemcitabine treatment of pancreatic cancer. *Mol Ther*. 2008; 16(2):252–260. [PubMed: 17984977]
62. Cozzi PJ, Wang J, Delprado W, Perkins AC, Allen BJ, Russell PJ, Li Y. MUC1, MUC2, MUC4, MUC5AC and MUC6 expression in the progression of prostate cancer. *Clin Exp Metastasis*. 2005; 22(7):565–573. [PubMed: 16475027]
63. Hinoda Y, Ikematsu Y, Horinochi M, Sato S, Yamamoto K, Nakano T, Fukui M, Suehiro Y, Hamanaka Y, Nishikawa Y, Kida H, Waki S, Oka M, Imai K, Yonezawa S. Increased expression of MUC1 in advanced pancreatic cancer. *J Gastroenterol*. 2003; 38(12):1162–1166. [PubMed: 14714254]
64. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS. Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res*. 1993; 53(3):641–651. [PubMed: 7678777]

65. Nagai S, Takenaka K, Sonobe M, Ogawa E, Wada H, Tanaka F. A novel classification of MUC1 expression is correlated with tumor differentiation and postoperative prognosis in non-small cell lung cancer. *J Thorac Oncol*. 2006; 1(1):46–51. [PubMed: 17409826]
66. Khodarev NN, Pitroda SP, Beckett MA, MacDermed DM, Huang L, Kufe DW, Weichselbaum RR. MUC1-induced transcriptional programs associated with tumorigenesis predict outcome in breast and lung cancer. *Cancer Res*. 2009; 69(7):2833–2837. [PubMed: 19318547]
67. Yin L, Li Y, Ren J, Kuwahara H, Kufe D. Human MUC1 carcinoma antigen regulates intracellular oxidant levels and the apoptotic response to oxidative stress. *J Biol Chem*. 2003; 278(37):35458–35464. [PubMed: 12826677]
68. Abe M, Kufe D. Structural analysis of the DF3 human breast carcinoma-associated protein. *Cancer Res*. 1989; 49(11):2834–2839. [PubMed: 2470498]
69. Li Y, Liu D, Chen D, Kharbanda S, Kufe D. Human DF3/MUC1 carcinoma-associated protein functions as an oncogene. *Oncogene*. 2003; 22(38):6107–6110. [PubMed: 12955090]
70. Fessler SP, Wotkowicz MT, Mahanta SK, Bamdad C. MUC1* is a determinant of trastuzumab (Herceptin) resistance in breast cancer cells. *Breast cancer research and treatment*. 2009
71. Walsh MD, Luckie SM, Cummings MC, Antalis TM, McGuckin MA. Heterogeneity of MUC1 expression by human breast carcinoma cell lines in vivo and in vitro. *Breast Cancer Res Treat*. 1999; 58(3):255–266. [PubMed: 10718487]
72. Engelmann K, Shen H, Finn OJ. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. *Cancer Res*. 2008; 68(7):2419–2426. [PubMed: 18381450]
73. Hikita ST, Kosik KS, Clegg DO, Bamdad C. MUC1* mediates the growth of human pluripotent stem cells. *PLoS One*. 2008; 3(10):e3312. [PubMed: 18833326]
74. Chen L, Chen D, Manome Y, Dong Y, Fine HA, Kufe DW. Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter. *J Clin Invest*. 1995; 96(6):2775–2782. [PubMed: 8675647]
75. Hernandez-Alcoceba R, Pihlaja M, Nunez G, Clarke MF. Evaluation of a new dual-specificity promoter for selective induction of apoptosis in breast cancer cells. *Cancer Gene Ther*. 2001; 8(4):298–307. [PubMed: 11393283]
76. Hernandez-Alcoceba R, Pihlaja M, Qian D, Clarke MF. New oncolytic adenoviruses with hypoxia- and estrogen receptor-regulated replication. *Hum Gene Ther*. 2002; 13(14):1737–1750. [PubMed: 12396626]
77. Kurihara T, Brough DE, Kovesdi I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J Clin Invest*. 2000; 106(6):763–771. [PubMed: 10995787]
78. Abe M, Kufe D. Characterization of cis-acting elements regulating transcription of the human DF3 breast carcinoma-associated antigen (MUC1) gene. *Proc Natl Acad Sci U S A*. 1993; 90(1):282–286. [PubMed: 8419933]
79. Kovarik A, Peat N, Wilson D, Gendler SJ, Taylor-Papadimitriou J. Analysis of the tissue-specific promoter of the MUC1 gene. *J Biol Chem*. 1993; 268(13):9917–9926. [PubMed: 8387509]
80. Kovarik A, Lu PJ, Peat N, Morris J, Taylor-Papadimitriou J. Two GC boxes (Sp1 sites) are involved in regulation of the activity of the epithelium-specific MUC1 promoter. *J Biol Chem*. 1996; 271(30):18140–18147. [PubMed: 8663395]
81. Aghi M, Hochberg F, Breakefield XO. Prodrug activation enzymes in cancer gene therapy. *J Gene Med*. 2000; 2(3):148–164. [PubMed: 10894261]
82. Rigg A, Sikora K. Genetic prodrug activation therapy. *Mol Med Today*. 1997; 3(8):359–366. [PubMed: 9269689]
83. Chen L, Waxman DJ. Cytochrome P450 gene-directed enzyme prodrug therapy (GDEPT) for cancer. *Curr Pharm Des*. 2002; 8(15):1405–1416. [PubMed: 12052216]
84. Jounaidi Y. Cytochrome P450-based gene therapy for cancer treatment: from concept to the clinic. *Curr Drug Metab*. 2002; 3(6):609–622. [PubMed: 12369889]
85. Ma J, Waxman DJ. Collaboration between hepatic and intratumoral prodrug activation in a P450 prodrug-activation gene therapy model for cancer treatment. *Mol Cancer Ther*. 2007; 6(11):2879–2890. [PubMed: 17989319]

86. Altaner C. Prodrug cancer gene therapy. *Cancer Lett.* 2008; 270(2):191–201. [PubMed: 18502571]
87. Schepelmann S, Ogilvie LM, Hedley D, Friedlos F, Martin J, Scanlon I, Chen P, Marais R, Springer CJ. Suicide gene therapy of human colon carcinoma xenografts using an armed oncolytic adenovirus expressing carboxypeptidase G2. *Cancer Res.* 2007; 67(10):4949–4955. [PubMed: 17510425]
88. Stubdal H, Perin N, Lemmon M, Holman P, Bauzon M, Potter PM, Danks MK, Fattaey A, Dubensky T, Johnson L. A prodrug strategy using ONYX-015-based replicating adenoviruses to deliver rabbit carboxylesterase to tumor cells for conversion of CPT-11 to SN-38. *Cancer Res.* 2003; 63(20):6900–6908. [PubMed: 14583489]
89. Dachs GU, Tupper J, Tozer GM. From bench to bedside for gene-directed enzyme prodrug therapy of cancer. *Anticancer Drugs.* 2005; 16(4):349–359. [PubMed: 15746571]
90. Both GW. Recent progress in gene-directed enzyme prodrug therapy: an emerging cancer treatment. *Curr Opin Mol Ther.* 2009; 11(4):421–432. [PubMed: 19649987]
91. Portsmouth D, Hlavaty J, Renner M. Suicide genes for cancer therapy. *Mol Aspects Med.* 2007; 28(1):4–41. [PubMed: 17306358]
92. Chen L, Waxman DJ. Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res.* 1995; 55(3):581–589. [PubMed: 7834628]
93. Chen L, Waxman DJ, Chen D, Kufe DW. Sensitization of human breast cancer cells to cyclophosphamide and ifosfamide by transfer of a liver cytochrome P450 gene. *Cancer Res.* 1996; 56(6):1331–1340. [PubMed: 8640822]
94. Jounaidi Y, Hecht JE, Waxman DJ. Retroviral transfer of human cytochrome P450 genes for oxazaphosphorine-based cancer gene therapy. *Cancer Res.* 1998; 58(19):4391–4401. [PubMed: 9766669]
95. Braybrooke JP, Slade A, Deplanque G, Harrop R, Madhusudan S, Forster MD, Gibson R, Makris A, Talbot DC, Steiner J, White L, Kan O, Naylor S, Carroll MW, Kingsman SM, Harris AL. Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. *Clin Cancer Res.* 2005; 11(4):1512–1520. [PubMed: 15746054]
96. Pawlik TM, Nakamura H, Mullen JT, Kasuya H, Yoon SS, Chandrasekhar S, Chioocca EA, Tanabe KK. Prodrug bioactivation and oncolysis of diffuse liver metastases by a herpes simplex virus 1 mutant that expresses the CYP2B1 transgene. *Cancer.* 2002; 95(5):1171–1181. [PubMed: 12209705]
97. Salmons B, Lohr M, Gunzburg WH. Treatment of inoperable pancreatic carcinoma using a cell-based local chemotherapy: results of a phase I/II clinical trial. *J Gastroenterol.* 2003; 38(Suppl 15): 78–84. [PubMed: 12698877]
98. Schwartz PS, Waxman DJ. Cyclophosphamide induces caspase 9-dependent apoptosis in 9L tumor cells. *Mol Pharmacol.* 2001; 60(6):1268–1279. [PubMed: 11723234]
99. Both GW. Gene-directed enzyme prodrug therapy for cancer: a glimpse into the future? *Discov Med.* 2009; 8(42):97–103. [PubMed: 19833053]
100. Schaack J, Schedl P, Shenk T. Topoisomerase I and II cleavage of adenovirus DNA in vivo: both topoisomerase activities appear to be required for adenovirus DNA replication. *J Virol.* 1990; 64(1):78–85. [PubMed: 2152835]
101. Painter RG, Lanson NA Jr, Jin Z, Park F, Wang G. Conditional expression of a suicide gene by the telomere reverse transcriptase promoter for potential post-therapeutic deletion of tumorigenesis. *Cancer Sci.* 2005; 96(9):607–613. [PubMed: 16128746]
102. Waxman DJ, Schwartz PS. Harnessing apoptosis for improved anticancer gene therapy. *Cancer Res.* 2003; 63(24):8563–8572. [PubMed: 14695163]
103. Clem RJ, Fechheimer M, Miller LK. Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science.* 1991; 254(5036):1388–1390. [PubMed: 1962198]
104. Stennicke HR, Ryan CA, Salvesen GS. Reprieve from execution: the molecular basis of caspase inhibition. *Trends Biochem Sci.* 2002; 27(2):94–101. [PubMed: 11852247]

105. Schwartz PS, Chen CS, Waxman DJ. Enhanced bystander cytotoxicity of P450 gene-directed enzyme prodrug therapy by expression of the antiapoptotic factor p35. *Cancer Res.* 2002; 62(23): 6928–6937. [PubMed: 12460909]
106. Doloff JC, Su T, Waxman DJ. Adenoviral delivery of pan-caspase inhibitor p35 enhances bystander killing by P450 gene-directed enzyme prodrug therapy using cyclophosphamide+ *BMC Cancer.* 2010; 10:487. [PubMed: 20836875]
107. Jounaidi Y, Waxman DJ. Use of replication-conditional adenovirus as a helper system to enhance delivery of P450 prodrug-activation genes for cancer therapy. *Cancer Res.* 2004; 64(1):292–303. [PubMed: 14729637]
108. McCormick F. Cancer-specific viruses and the development of ONYX-015. *Cancer Biol Ther.* 2003; 2(4 Suppl 1):S157–S160. [PubMed: 14508094]
109. Jounaidi Y, Chen CS, Veal GJ, Waxman DJ. Enhanced antitumor activity of P450 prodrug-based gene therapy using the low Km cyclophosphamide 4-hydroxylase P450 2B11. *Mol Cancer Ther.* 2006; 5(3):541–555. [PubMed: 16546968]
110. Subramanian T, Vijayalingam S, Chinnadurai G. Genetic identification of adenovirus type 5 genes that influence viral spread. *J Virol.* 2006; 80(4):2000–2012. [PubMed: 16439556]
111. Francis P, Fernebro J, Eden P, Laurell A, Rydholm A, Domanski HA, Breslin T, Hegardt C, Borg A, Nilbert M. Intratumor versus intertumor heterogeneity in gene expression profiles of soft-tissue sarcomas. *Genes Chromosomes Cancer.* 2005; 43(3):302–308. [PubMed: 15834945]
112. Nagasaki K, Miki Y. Gene expression profiling of breast cancer. *Breast Cancer.* 2006; 13(1):2–7. [PubMed: 16518056]
113. Leedham SJ, Wright NA. Expansion of a mutated clone: from stem cell to tumour. *J Clin Pathol.* 2008; 61(2):164–171. [PubMed: 17468295]
114. Mimeault M, Hauke R, Mehta PP, Batra SK. Recent advances in cancer stem/progenitor cell research: therapeutic implications for overcoming resistance to the most aggressive cancers. *J Cell Mol Med.* 2007; 11(5):981–1011. [PubMed: 17979879]
115. Tredan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst.* 2007; 99(19):1441–1454. [PubMed: 17895480]
116. Yan P, Benhattar J, Coindre JM, Guillou L. Telomerase activity and hTERT mRNA expression can be heterogeneous and does not correlate with telomere length in soft tissue sarcomas. *Int J Cancer.* 2002; 98(6):851–856. [PubMed: 11948462]
117. Kleinschmidt-DeMasters BK, Hashizumi TL, Sze CI, Lillehei KO, Shroyer AL, Shroyer KR. Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling. *J Clin Pathol.* 1998; 51(4): 284–293. [PubMed: 9659240]
118. Kleinschmidt-DeMasters BK, Shroyer AL, Hashizumi TL, Evans LC, Markham N, Kindt G, Shroyer KR. Part I. Telomerase levels in human metastatic brain tumors show four-fold logarithmic variability but no correlation with tumor type or interval to patient demise. *J Neurol Sci.* 1998; 161(2):116–123. [PubMed: 9879692]
119. Kleinschmidt-DeMasters BK, Evans LC, Bitter MA, Shroyer AL, Shroyer KR. Part II. Telomerase expression in cerebrospinal fluid specimens as an adjunct to cytologic diagnosis. *J Neurol Sci.* 1998; 161(2):124–134. [PubMed: 9879693]
120. Wullich B, Rohde V, Oehlenschlaeger B, Bonkhoff H, Ketter R, Zwergel T, Sattler HP. Focal intratumoral heterogeneity for telomerase activity in human prostate cancer. *J Urol.* 1999; 161(6): 1997–2001. [PubMed: 10332489]
121. Carey LA, Hedican CA, Henderson GS, Umbricht CB, Dome JS, Varon D, Sukumar S. Careful histological confirmation and microdissection reveal telomerase activity in otherwise telomerase-negative breast cancers. *Clin Cancer Res.* 1998; 4(2):435–440. [PubMed: 9516933]
122. Youssef N, Paradis V, Ferlicot S, Bedossa P. In situ detection of telomerase enzymatic activity in human hepatocellular carcinogenesis. *J Pathol.* 2001; 194(4):459–465. [PubMed: 11523054]
123. Hao X, Sun B, Hu L, Lahdesmaki H, Dunmire V, Feng Y, Zhang SW, Wang H, Wu C, Wang H, Fuller GN, Symmans WF, Shmulevich I, Zhang W. Differential gene and protein expression in primary breast malignancies and their lymph node metastases as revealed by combined cDNA

microarray and tissue microarray analysis. *Cancer*. 2004; 100(6):1110–1122. [PubMed: 15022276]

124. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN. Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. *Oncogene*. 2006; 25(1): 61–69. [PubMed: 16170363]
125. Yan P, Benhattar J, Seelentag W, Stehle JC, Bosman FT. Immunohistochemical localization of hTERT protein in human tissues. *Histochem Cell Biol*. 2004; 121(5):391–397. [PubMed: 15138842]
126. Merlo GR, Siddiqui J, Cropp CS, Liscia DS, Lidereau R, Callahan R, Kufe DW. Frequent alteration of the DF3 tumor-associated antigen gene in primary human breast carcinomas. *Cancer Res*. 1989; 49(24 Pt 1):6966–6971. [PubMed: 2582438]
127. Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, Porten M, Herrmann I, Ristimäki A, Virkkunen P, Tarkkanen M, Hakkarainen T, Kanerva A, Rein D, Pesonen S, Hemminki A. Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. *Cancer Res*. 2008; 68(14):5533–5539. [PubMed: 18632604]
128. Ribacka C, Pesonen S, Hemminki A. Cancer, stem cells, and oncolytic viruses. *Ann Med*. 2008; 40(7):496–505. [PubMed: 18608120]
129. Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, Bedell C, Adams N, Zhang YA, Maples PB, Chen S, Pappen B, Burke J, Ichimaru D, Urata Y, Fujiwara T. A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol Ther*. 2010; 18(2):429–434. [PubMed: 19935775]
130. Taki M, Kagawa S, Nishizaki M, Mizuguchi H, Hayakawa T, Kyo S, Nagai K, Urata Y, Tanaka N, Fujiwara T. Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD'). *Oncogene*. 2005; 24(19):3130–3140. [PubMed: 15735729]
131. Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther*. 2004; 15(12): 1157–1166. [PubMed: 15684693]
132. Zhu J, Huang X, Yang Y. A critical role for type I IFN-dependent NK cell activation in innate immune elimination of adenoviral vectors in vivo. *Mol Ther*. 2008; 16(7):1300–1307. [PubMed: 18443600]
133. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses. *Immunol Rev*. 2000; 174:35–46. [PubMed: 10807505]
134. Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. *Gene Ther*. 2010; 17(3):295–304. [PubMed: 19907498]
135. Hedley SJ, Chen J, Mountz JD, Li J, Curiel DT, Korokhov N, Kovesdi I. Targeted and shielded adenovectors for cancer therapy. *Cancer Immunol Immunother*. 2006; 55(11):1412–1419. [PubMed: 16612598]
136. O'Riordan CR, Lachapelle A, Delgado C, Parkes V, Wadsworth SC, Smith AE, Francis GE. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther*. 1999; 10(8):1349–1358. [PubMed: 10365665]
137. De Geest B, Snoeys J, Van Linthout S, Lievens J, Collen D. Elimination of innate immune responses and liver inflammation by PEGylation of adenoviral vectors and methylprednisolone. *Hum Gene Ther*. 2005; 16(12):1439–1451. [PubMed: 16390275]
138. Power AT, Bell JC. Cell-based delivery of oncolytic viruses: a new strategic alliance for a biological strike against cancer. *Mol Ther*. 2007; 15(4):660–665. [PubMed: 17264852]
139. Power AT, Wang J, Falls TJ, Paterson JM, Parato KA, Lichty BD, Stojdl DF, Forsyth PA, Atkins H, Bell JC. Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. *Mol Ther*. 2007; 15(1):123–130. [PubMed: 17164783]
140. Garcia-Castro J, Martinez-Palacio J, Lillo R, Garcia-Sanchez F, Alemany R, Madero L, Bueren JA, Ramirez M. Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors. *Cancer Gene Ther*. 2005; 12(4):341–349. [PubMed: 15650763]

141. Warram JM, Sorace AG, Saini R, Borovjagin AV, Hoyt K, Zinn KR. Systemic delivery of a breast cancer-detecting adenovirus using targeted microbubbles. *Cancer Gene Ther.* 2012

Figure 1. Targeting tumor heterogeneity

A) Core DF3/Mucin1 (DF3/MUC1) and human telomerase (hTERT) promoters utilized to regulate adenoviral replication. These promoters use different transcription factor binding sites and distinct mechanisms of transcriptional activation, i.e., the presence or absence of a canonical TATA box or CpG methylation. Nucleotide +1, transcription start site. B) Multiple promoters regulating adenoviral replication may help ensure viral replication and increased anti-tumor activity in a heterogeneous tumor cell population. Since DF3/MUC1 is overexpressed in ~75% of all human solid tumors [62–66] and the hTERT gene is activated in 85–90% of tumor tissues [45], the combined use of the corresponding two promoters to express adenoviral E1A will help ensure successful induction of adenoviral replication in a heterogeneous tumor cell population, where either one, or both promoters is active.

A) Conventional Treatment

B) Adenoviral Gene delivery + Drug Treatment

Figure 2. Gene-directed prodrug-activating enzyme therapy (GDEPT) using adenoviral vectors
A) Traditional chemotherapy involves systemic drug delivery, often resulting in non-specific, toxic side effects to multiple host tissues. B) Introduction of the capacity for intratumoral prodrug activation, *via* adenovirus-mediated gene therapy, may allow for use of a lower dose of the anti-cancer prodrug, thereby decreasing non-specific systemic side effects while increasing local, intratumoral anti-tumor activity.

A) Non-Replicative virus alone

B) Replication-deficient adenovirus + Replication-competent helper

Figure 3. Enhancing GDEPT bystander activity by concomitant delivery of the pan-caspase inhibitor p35

A) Adenoviral gene-directed enzyme-prodrug therapy (GDEPT) facilitates chemotherapeutic prodrug activation and tumor cell kill. However, the adenovirally transduced tumor cell may die quickly due to its exposure to high local concentrations of active drug metabolites. B) The adenovirally transduced tumor factory cell may be protected from caspase-dependent apoptotic cell death by delivery of the pan-caspase inhibitor p35. The increased longevity of these factory cells that results prolongs their ability to metabolize an inactive chemotherapeutic prodrug into its active, cytotoxic metabolites, thereby enhancing anti-tumor activity. p35-infected tumor cells eventually die by a caspase-independent mechanism.

A) Adenoviral Gene Therapy + Prodrug

B) GDEPT + p35 (caspase inhibitor) + Prodrug

Figure 4. Adenoviral helper virus effect

A) Administration of a replication-deficient adenovirus leads to successful gene transfer to those (few) tumor cells directly infected by the virus. B) Infection of tumor cells with a replication-defective virus carrying a prodrug-activating gene (as in A) in combination with a replication-competent adenovirus facilitates spread of the viral infection to include secondary sites of infection. The replicating virus serves as a helper virus that supplies all of the required replication and repackaging machinery *in trans*, leading to spread of both adenoviral vectors in the treated tumor cell population.